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# Silk-Fiber Immobilized Lipase-Catalyzed Hydrolysis of Emulsified Sunflower Oil

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**Abstract** Lipase was immobilized in silk fibers through glutaraldehyde cross-linking to a maximum loading of 59 U/g silk-fiber and the immobilized lipase was utilized for the hydrolysis of sunflower oil (*Helianthus annuus*). The hydrolytic activity of the lipase, which was poor in biphasic oil in water system, was increased significantly when the sunflower oil was emulsified in aqueous medium. The hydrolytic activities of the immobilized lipase were 48.73±1.26 U, 36.11±0.96 U, and nil when the substrate sunflower oil was used as emulsion created by a rhamnolipid biosurfactant, Triton X100, and ultrasonication, respectively. Although the efficiency of the immobilized lipase was less than 12% than the corresponding free lipase, the immobilized lipase could be reused for the biosurfactant-mediated hydrolysis of sunflower oil up to third cycle of the reaction. The yield of the fatty acids in the second, third, and fourth cycles were 49.45%, 22.91%, and 5.09%, respectively, of the yield obtained in the first cycle.

**Keywords** Sunflower oil · Lipase · Immobilization · Fatty acid · Biosurfactant · Silk fiber

## Introduction

Hydrolysis of fats and oils is a well-known important activity in oleo chemical industries for the production of fatty acid, which is a renewable feedstock for industrial-scale processing to give value-added products for food, beverages, cosmetics, and medicinal applications [1, 2]. In the fatty acid industry, hydrolysis of fats and oils is usually accomplished by a high-temperature steam treatment method that operates with super-heated steam and high pressure

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[3]. This high-temperature process is energy intensive and causes extensive degradation of the produced fatty acids. There is a growing interest to replace this chemical method with enzymatic methods as the later methods are normally operated under mild conditions in an energy efficient manner. The lipase is a lipolytic enzyme that functions at oil–water interface and generates less undesirable by products during hydrolysis of fats and, hence, its application potential as catalysts for hydrolysis of oil and fat is growingly focused [4–7]. Since interfacial activation is essential for catalytic activity of lipase [8], various surface-active agents are employed to emulsify the fat in aqueous phase for enhancing the hydrolytic activity of lipases. However, majority of the surface-active agents utilized for the hydrolysis are synthetic and non-biodegradable in nature, thus, the effluent generated from the down-stream processing may cause environmental pollution if preventive measure is not taken. Biosurfactants are usually biodegradable surface-active agents. One of the focuses of our work presented here is to utilize biosurfactant for the lipase-catalyzed hydrolysis of vegetable oil.

Application of immobilized enzyme for industrial process is a well-known fact that contributes to the process economy through reuse of the enzyme. Potential methods and support materials for immobilization of lipases have been reviewed [9]. Further on this line we are describing here a potential biodegradable support system for the immobilization of lipase to be used for the hydrolysis of a vegetable oil.

## Materials and Methods

### Materials

Sunflower oil (saturates 12%, polyunsaturates 60%, density 0.91 g/cc at 30 °C) was purchased from the local market. Lipase (triacylglycerin lipase: E.C. 3.1.1.3) from *Aspergillus niger* was obtained from HIMEDIA, India. Glutaraldehyde was purchased from Merck, India. Woven Muga Silk fiber produced by *Antherea assamensis* was purchased from Sualkuchi (Silk village of Assam, India) and used as support system for immobilization of the lipase.

### Lipase Assay

Free or immobilized lipase activity was determined by glycerol tributyrin-polyvinyl alcohol emulsion method, which is a partial modification of the method described by Ibrahim et al. [10]. One percent (*w*/*v*) polyvinyl alcohol was dissolved in 50 ml water by heating at 50 °C–60 °C. Two percent (*v*/*v*) glycerol tributyrin was dissolved in the above solution and sonicated to form an emulsion. Four milliliters of this emulsion solution was mixed with 6 ml solution of sodium phosphate buffer (0.1 M) pH 7.0 and a suitable quantity of the lipase immobilized silk fiber suspended in a 250 ml of conical flask and incubated for 20 min in orbital shaker at 30 °C and 200 rpm. The reaction was terminated by adding 20 ml solution of acetone–ethanol in the ratio 1:1. Free fatty acid liberated was titrated against 0.02 N sodium hydroxide solution using phenolphthalein as indicator. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μmol of fatty acid per minute at 30 °C.

### Immobilization of Lipase

A 0.1 g of commercial lipase was dissolved in 100 ml of 50 mM potassium phosphate buffer of pH 6.5. Woven silk fibers were cut into 20×20 mm pieces (dry weight 0.038 g) and washed

successively with 0.04 N NaOH solution and 0.1 M sodium phosphate buffer of pH 7.0. The cleaned and washed fibers were then suspended in the above lipase solution. The suspended solution was then mixed with suitable concentration of glutaraldehyde and kept at room temperature (25 °C) for minimum 24 h. The activity of the cross-linked enzyme on the silk fiber was assayed after washing the fiber pieces with 50 mM sodium phosphate buffer of pH 8.0 for several times. The lipase immobilized silk fibers were stored at 4 °C for further use.

## Isolation and Characterization of The Biosurfactant

A biosurfactant was isolated and purified from the culture broth of an *n*-hexadecane grown *Pseudomonas aeruginosa* strain SS 01 isolated following the similar procedure described by Finnerty et al. [11]. The media composition and growth condition for harvesting the biosurfactant were similar to our earlier report [12]. Analysis of the glycolipid fraction obtained from the silicic acid column chromatography by thin-layer chromatography (TLC) reveals one major component and several minor components. The major fraction, eluted by hexane, ethyl acetate, and methanol at a ratio of 3:7:10 through the silicic acid column was collected. The solvent was removed by vacuum rotary evaporator and the biosurfactant was collected as dry brown viscous material. The chemical characteristic of the biosurfactant as rhamnolipid was established by analyzing the purified biosurfactant through FT-IR (Spectrum One, Perkin Elmer) and Q-TOF Premier Mass Spectrometer (M/s Waters). Samples were obtained in negative ionization mode for electrospray ionization mass-spectrometry (ESI-MS) study of the pure biosurfactant. The samples were prepared in 1:1 (*v*/*v*) HPLC grade methanol/water with 0.01% NaOH in 10 pg/μl concentration. The samples were filtered through 0.02 μ filter paper and sonicated in ultrasonic bath for 20 min and then injected (2–5 μl) into the source at a rate of 10 μl/min using a carrier solvent of water–methanol 1:1 (*v*/*v*) with 0.025% sodium hydroxide. The sampling cone voltage was set at 40 V. ESI-MS was operated at spray voltage 3.5 kV, and the heated capillary temperature was maintained at 120 °C. Nitrogen was used as nebulizing and drying gas with flow rate of 2.5 and 8 L/min, respectively. Data were acquired over the mass range of m/z 200 to 1,000. Rhamnose sugar was estimated by established orcinol–sulfuric acid methods.

## Lipase-Catalyzed Hydrolysis of Sunflower Oil

The reaction mixture consists of 8 ml of 0.1 M phosphate buffer of pH 7.2, 1 ml of sunflower oil, 1 ml of free lipase (10 mg/ml corresponding to 160 U/ml as per the unit of the powdered lipase mentioned by the supplier) solution or an equivalent quantity (in terms of protein) of the immobilized lipase, and Triton X100 or biosurfactant at a concentration of 20% higher than their respective critical micelle concentration (CMC). The hydrolysis was carried out at 30 °C, 150 rpm for 6 h. The reaction was stopped by removing the immobilized lipase or with 20 ml of acetone–ethanol mixture (1:1 *v*/*v* ratio) for powdered lipase. Amount of free fatty acid liberated on hydrolysis was monitored by titrating the supernatant against 0.02 N NaOH solution using phenolphthalein as indicator. After each cycle of operation the silk-fiber was thoroughly washed with alkaline (pH 8.0) sodium phosphate buffer to use the immobilized lipase for the next cycle of the reaction.

## Statistical Analysis

Results obtained are the mean of three or more determinations. Analysis of variance was carried out on all data at $p<0.05$.

## Results and Discussion

### Immobilization of the Lipase

The lipase was immobilized on silk fiber through cross-linking with glutaraldehyde. The loading of the lipase on the fiber was optimized at room temperature (25 °C) by varying any of the parameters namely, concentration of glutaraldehyde, and lipase treatment time, while keeping the other parameter constant. Concentration of glutaraldehyde was found to be critical on the activity of the loaded enzyme. The optimum concentration of glutaraldehyde was found to be 5% (*v*/*v*) as shown in Fig. 1. The activity of the enzyme in the fiber declined beyond this concentration of glutaraldehyde. On prolong incubation of the reaction mixture beyond 24 h with the said concentration of glutaraldehyde the enzyme loading was marginally increased and it was reached to a maximum limit of 59.16 U/g silk fiber at 27 h of incubation.

The morphological change of the silk fibers upon immobilization of the lipase was also detected by scanning electron microscopy. For clarity of the morphological differences before and after immobilization, the resolution was captured for single silk fiber as shown in Fig. 2A,B. White patches in the fiber structure after lipase treatment of the silk fibers, which was not visible before lipase treatment, was also observed (Fig. 2B).

The storage stability of the immobilized lipase was investigated and found to be stable for months. The activity of the lipase was almost retained at 4 °C while stored at least for 2 months. Although the storage stability of both the immobilized and free lipase was declined at room temperature, the relative stability of the immobilized lipase was significantly higher than the free lipase. The decreasing of lipase activity per week for the immobilized and equivalent free lipase was 2.83 U (from the initial loading of 59 U/g of the silk fiber) and 2.91 U, respectively.

### Analysis of Biosurfactant

FT-IR analysis of the purified biosurfactant in KBr showed strong broad absorption at 3,419 $cm^{-1}$ indicating the presence of −OH group in the molecule, while, absorption at 1,651 $cm^{-1}$ indicates the stretching mode for >CO–O bond. The bands at 2,925 $cm^{-1}$, 2,854 $cm^{-1}$, 1,470 $cm^{-1}$, and at 1,378 $cm^{-1}$ reflect aliphatic chains ($-CH_3$, $-CH_{2-}$) of the fraction. The absorption at 1,740 $cm^{-1}$ was due to lactone carbonyl absorption, indicating that the product contains aliphatic hydrocarbon as well as sugar-like moiety. No

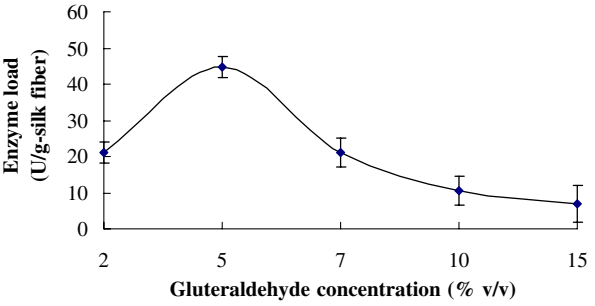


**Fig. 1** Optimum concentration of glutaraldehyde for enzyme loading on the silk fiber at 24 h of incubation

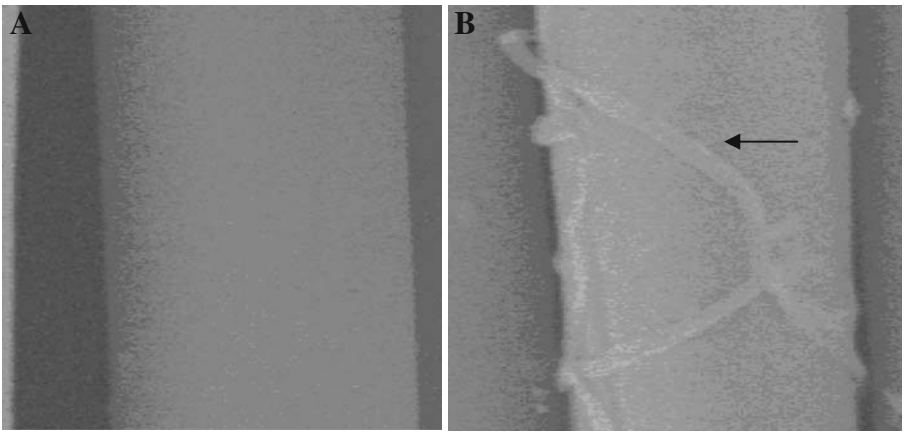


**Fig. 2** SEM image of Silk fiber: **A** before and **B** after immobilization of lipase (magnification 1.75 KX)

characteristic bands corresponding to the −N-H stretching and deformation mode was detected in the spectra, thus, the presence of peptide or amino moiety in the biosurfactant molecule is ruled out. Chemical analysis for sugar showed the presence of rhamnose sugar in the purified biosurfactant. ESI-MS analysis showed molecular ion peak (m/z) at 701.98. This value is found to be similar for the rhamnolipid structure Rha-Rha-C12:1-C12 and Rha-Rha-C10-C14:1 reported by Haba et al. [13]. Above findings suggested rhamnolipid nature of the isolated biosurfactant.

## Hydrolysis of Sunflower Oil with Lipase

Lipase catalyzed hydrolysis of sunflower oil in two phase system (oil/water) was studied using free or silk-fiber immobilized lipase (Table 1). The hydrolytic activities of both the free and immobilized lipases were found to be poor even after changing the various operational parameters like, temperature, shaking speed, and substrate–catalyst ratio, thus, appeared unsuitable for the process. The poor catalytic activity of the lipase was attributed to the low surface to volume ratio of the oil in the reaction mixture resulting in low substrate availability for the catalysis as well as lack of interfacial activation of lipase in this two-phase system. We proposed to promote this hydrolysis in oil in water emulsion system. A non-ionic surfactant, Triton X100, ultrasonic vibration, and the rhamnolipid biosurfactant were used to generate the emulsion, which were then used for lipase-catalyzed hydrolysis of sunflower oil. The concentration of the Triton X100 and rhamnolipid were used at a

**Table 1** Hydrolytic activity of the free and immobilized lipase under different condition of the sunflower oil.

| Condition of oil in the medium | Activity (U) | | |
|---|---|---|---|
| | Free lipase | Immobilized lipase | Retention (%) |
| O/W with or without ultrasonication | Trace (<5) | Nil | Nil |
| O/W emulsion using Triton X100 | 48.01 | 36.11 | 75.20 |
| O/W emulsion using biosurfactant | 59.65 | 48.73 | 87.50 |

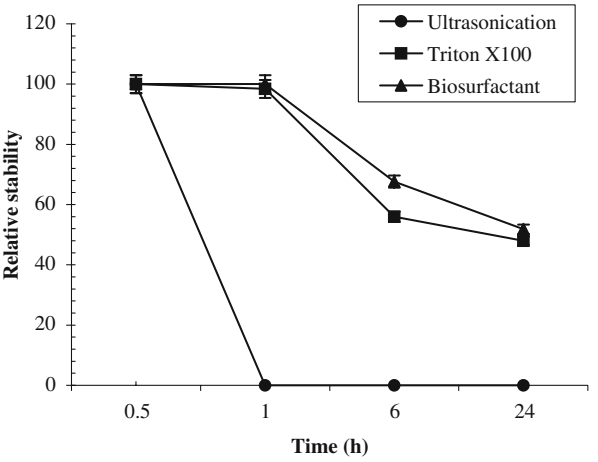


**Fig. 3** Stability of emulsions generated by utrasonication, biosurfactant, and Triton X100

concentration of 20% higher than their respective critical micelle concentrations of $0.2\times10^{-3}$ M and $0.0213\times10^{-3}$ M, respectively, to ensure the sufficient emulsification of the oil used for the hydrolytic reaction. The stability of the emulsions created by these chemical surfactant, biosurfactant, and ultrasonication was studied at room temperature and found that the stability of the emulsions generated by the biosurfactant and Triton X100 were nearly equivalent up to 24 h of incubation (Fig. 3). However, when these emulsions were kept at 4 °C for a week the stability of the Triton X100-mediated emulsion was largely depleted whereas, the stability of the biosurfactant-mediated emulsion was retained even more than 50% of the original value.

## Activity and Stability of Lipase

The activity of the immobilized lipase was studied for the hydrolysis of emulsified sunflower oil created by using biosurfactant and Triton X100 separately (Table 1). The hydrolytic activity of the lipase was increased when the oil was emulsified in the reaction mixture, while this increase was higher in the biosurfactant-mediated emulsion of the oil than the Triton X100-mediated emulsion. The activity of the lipase was nearly void when ultrasonicated emulsion was used. The hydrolytic activity of the lipase for the oil emulsion created by the Triton X100 and biosurfactant were 36.11±0.96 U and 48.73±1.26 U, respectively. Thus, nearly, 25.9% higher activity of the immobilized lipase was demonstrated in biosurfactant-mediated emulsion over Triton X100-mediated emulsion.

The reason for enhanced catalytic activity of the lipase for the hydrolysis of biosurfactant-mediated emulsion is uncertain yet. However, it is suggested that this surfactant from the biological sources might promote better interfacial microenvironment for enhanced activity of the lipase. Further investigation on the interfacial microenvironment created by the biosurfactant is warranted to unveil the fact. The retention of activity of the immobilized lipase in Triton X100-mediated hydrolysis was nearly 12% less than the biosurfactant-mediated hydrolysis as evident from the data shown in Table 1. This implies the partial inhibitory effect of Triton X100 as compared to the effect of corresponding biosurfactant on the immobilized lipase-mediated hydrolysis of the oil.

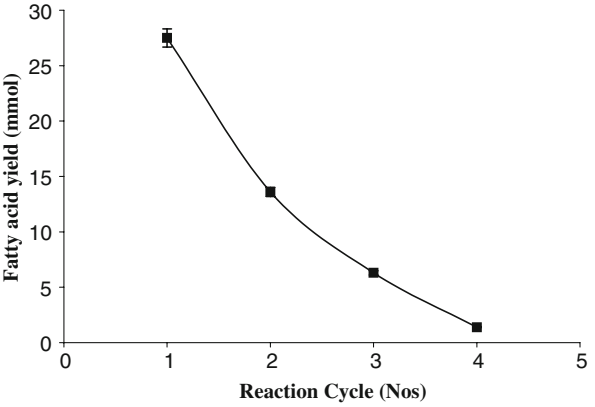


**Fig. 4** Yield of fatty acid during silk-fiber immobilized lipase catalyzed hydrolysis of sunflower oil in different reaction cycles

Reusability of Lipase

The reusability of the silk fiber-immobilized lipase for the hydrolysis of sunflower oil was studied by repeating the hydrolytic reactions with a single set of immobilized lipase (Fig. 4). Each reaction was carried out at 30 °C and 150 rpm for 6 h. After each cycle the fibers (each membrane of 20×20 mm sizes) were subjected to stringent washing with methanol and hexane in 1:1 ratio followed by sodium phosphate buffer of pH 8.0. The yield of the fatty acids in the second, third, and fourth cycles were 49.45%, 22.91%, and 5.09%, respectively, of the yield obtained in the first cycle, considering the yield 27.5 mmol in the first cycle as 100%. We observed that to regain sufficient activity after each cycle of the operation the immobilized lipase required thorough washing with slightly alkaline (pH 8.0) sodium phosphate buffer. The reason is ascribed to the dissolution of the product fatty acids associated with the lipase by the alkaline buffer solution and thereby making the active sites of the lipase free for further catalysis.

## Conclusions

The silk fiber, which is a known biodegradable and renewable biomaterial, as support for the chemical immobilization of lipase is reported. The immobilized lipase was successfully used for the hydrolysis of sunflower oil for the production of fatty acids. This lipase catalyzed hydrolysis was found to occur effectively when the substrate sunflower oil was used as emulsion, mediated by either a chemical or a biological surface active agent, namely, Triton X100 and rhamnolipid, respectively. Further, we demonstrated that the rhamnolipid was a better surface-active agent than the Triton X100 for the hydrolysis as evident from the 25.9% higher lipase activity realized by using emulsion created by biosurfactant over Triton X100. Although, the efficiency of the immobilized lipase was nearly 12% less than the free lipase on the biosurfactant-mediated hydrolysis of sunflower oil, the immobilized lipase could be reused even up to the third reaction cycle in this biosurfactant-mediated conversion, thus, appeared as potential industrial biocatalytic process for hydrolysis of vegetable oil.

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